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Grainstacks at the End of Summer, Evening Effect was one of 25 canvases of wheat or haystacks Claude Monet painted between 1890 and 1891, which explored the effect of variations in point of view, time of day, season, and weather on their common theme. These works were as a group commercially successful and popular with both critics and the public. They marked a turning point in Monet’s career not only in this regard, but also for his having painted them in the fields surrounding a house he purchased that year in Giverny. They represent the beginning of a more grounded life there and what would become a frequent serial approach to his subjects. The painting on the cover follows the impressionist strategy of focusing on archetypal shape and color rather than detail, here the warm shades with which Monet imbues the estivating ricks. In this issue Nobles and Wong report on a modified, more time-efficient method of warm autoantibody adsorption.

David Moolten, MD
An \textit{AQP1} allele associated with Co(a−b−) phenotype

S. Vege, S. Nance, D. Kavitsky, X. Li, T. Horn, G. Meny, and C.M. Westhoff

The Colton (CO) blood group system consists of four antigens, Co\(^a\), Co\(^b\), Co3, and Co4, located on aquaporin-1 (\textit{AQP1}), with Co\(^a\) highly prevalent in all populations (99.8%). The Colton null phenotype, Co(a−b−), is very rare, and individuals with this phenotype lack the high-prevalence antigen Co3. To date, only six Co(a−b−) probands have been reported and five silencing alleles characterized. We identified an \textit{AQP1}-null allele in a white woman with anti-Co3 caused by deletion of a G at nucleotide 601 (nt601delG) that results in a frameshift and premature termination (Val201Stop). Available family members were tested for the allele. Although anti-Co3 has been associated with mild to severe hemolytic disease of the fetus and newborn, the antibody was not clinically significant as evidenced by a low titer and delivery of asymptomatic newborns with moderate to weakly positive direct antiglobulin tests for all four pregnancies. 


\textbf{Key Words:} Colton blood group, Co(a−b−), \textit{AQP1} Co\(_n\)ull, anti-Co3

The Colton (CO) blood group system consists of four antigens, Co\(^a\), Co\(^b\), Co3, and Co4, located on aquaporin-1 (\textit{AQP1}). The prevalence of Co\(^a\) is high in all populations (99.8%). The antithetical antigen, Co\(^b\), is found in approximately 10 percent of Europeans and 5 percent of Hispanics and less frequently in Japanese. Co3 is the high-prevalence antigen absent in Co(a−b−) individuals. The Co\(_n\)ull phenotype, Co(a−b−), Co:–3, is extremely rare. Co4 is a high-prevalence antigen that was reported in two samples that were initially thought to be phenotype Co(a−b−) with a possible anti-Co3. Further expression studies and reevaluation of antibody specificity indicated this antibody is associated with a new antigen, Co4.4,5 The \textit{AQP1} protein is a member of a large family of water transport channels expressed on the erythrocyte membrane. Individuals with the Co(a−b−) phenotype also lack AQP1 protein in other tissues. Although no severe clinical effects are associated with the null phenotype,\(^6\) it has been shown that such individuals, when in water-deprived conditions, have a limited ability to concentrate urine and have decreased pulmonary vascular permeability.\(^7\)

The \textit{AQP1} gene, consisting of four exons, is located on the short arm of chromosome 7 (7q14). The gene encodes a protein of 268 amino acids with a 28-kDa relative molecular mass. The Co\(^a\) and Co\(^b\) antigens correspond to a nucleotide (nt) 134C>T change, encoding an amino acid change at position 45 from alanine to valine.\(^8\) Only six probands with the rare Co(a−b−) phenotype have been reported, and five silencing alleles have been characterized: (1) deletion of most of exon 1 (CO*N.01),\(^9\) (2) insertion of T at nt 307 that results in a frameshift in the protein at Gly104 (CO*N.02),\(^9\) (3) an nt 576C>A change encoding Asn192Lys (CO*01N.03),\(^10\) (4) deletion of G at nt 232 causing a frameshift at amino acid 78 and a premature stop codon at position 119 (CO*01N.04),\(^11\) and (5) an nt 112C>T change encoding Pro38Ser (CO*01N.05).\(^12\) A homozygous 113C>T change (Pro38Leu) (CO*M.01) was found in an individual whose red blood cells (RBCs) typed Co(a−b−) with weak AQP1 expression (<1% of control) detected in RBC membrane immunoblots.\(^9\) Importantly, Co\(_n\)ull individuals have been reported to make clinically significant anti-Co3, causing mild to severe hemolytic disease of the fetus and newborn (HDFN).\(^11,13\) Transfusion of patients with anti-Co3 is difficult because of the rarity of the type. Autologous donation and testing of family members are strongly recommended. The American Rare Donor Program has one Co:–3 donor. The World Health Organization International Panel has two donors listed in Canada and nine units frozen in France (number of donors not known).

Here, we identify a Colton null allele in a woman whose RBCs type as Co(a−b−) with anti-Co3 in her serum. We report results of testing family members and describe the clinical management of her pregnancies.

\textbf{Case Report}

Anti-Co3 was identified in a 33-year-old gravida 2 para 1 white woman of European descent. Her RBCs typed Co(a−b−), Co:–3. She had one pregnancy 14 years before and no history of blood transfusions. Her first pregnancy was unremarkable. During the course of the second pregnancy, the anti-Co3 was identified and she donated two autologous directed units for her or her baby’s potential need. These units were divided into two aliquots each and frozen. Only two aliquots per unit could be made because of her low hemoglobin levels at the time of
the donations. A baby girl (child 2) was delivered by cesarean delivery at 34 weeks’ gestation. The baby’s direct antiglobulin test (DAT) was 1+. Neither the baby nor the mother required transfusion. Nearly 2 years later, she delivered another baby (child 3). This baby’s DAT was weakly positive. Two years after the third child, she delivered a fourth child (child 4), whose RBCs showed a microscopically weakly positive DAT. The third and fourth pregnancies were managed with the same protocol for autologous unit collection; neither required transfusion.

Materials and Methods

EDTA-anticoagulated whole blood samples were obtained from the proband, her husband, child 2, and child 3. A buccal swab sample was obtained from child 4.

Serologic testing was performed on the husband’s sample; no DNA was isolated for molecular analysis.

Serology

Antibody identification and RBC typing were performed using standard methods. Determination of antibody titers was performed with 6 percent albumin as a diluent in the proband’s serum at 4, 5, 6, 7, and 8 months’ gestation, before delivery, and 16 months after delivery of child 2. Samples were obtained during the third pregnancy at 4, 6, and 8 months’ estimated gestational age (EGA) and at the time of autologous donation in the fourth pregnancy.

Polymerase Chain Reaction Amplification and DNA Sequence Analysis

DNA was isolated with QIAamp Blood Mini Kit (QIAamp Blood Mini Kit, QIAGEN, Hilden, Germany). CO (AQP1) was amplified with previously published primers. Amplified polymerase chain reaction (PCR) products were purified (PCR Purification kit, Qiagen, Inc., Valencia, CA) and sequenced by the Children’s Hospital of Philadelphia Sequencing Facility. Primers used for PCR amplification and DNA sequence analysis are listed in Table 1. For some samples, PCR products were cloned (TOPO TA-cloning kit, Invitrogen, Carlsbad, CA), and plasmids were sequenced using vector primers. Sequences were aligned to reference sequence (GenBank accession #NM_198098) with ClustalX software (ClustalX, Science Foundation Ireland and University College Dublin, Dublin, Ireland).

Western Blot Analysis

RBC membranes were prepared by centrifugation of whole blood for 10 minutes and removal of plasma and the buffy coat. Packed RBCs were washed with phosphate-buffered saline (PBS) and lysed in 5 mM Tris HCl/0.1 mM EDTA, pH 7.5, on ice for 15 minutes. Cell membranes were washed four times with 5 mM Tris HCl/0.1 mM EDTA and one time with PBS by centrifugation at 35,000 g. Protein concentration was determined using Pierce BCA Protein Assay Kit, Thermo Scientific, Rockford, IL. RBC membrane protein (100 μg) was separated on 13.5 percent nonreducing sodium dodecylsulfate (SDS) polyacrylamide gel electrophoresis, transferred to nitrocellulose membrane, and incubated first with anti-Co3 and then with horseradish peroxidase–conjugated goat anti-human IgG. Anti-Co3 was a gift from Peter Agre. The blot was visualized by chemiluminescence (ECL, Invitrogen).

RBC Antigen Typing and Antibody Titrations

The proband’s RBCs typed as group A, D+, C+, E−, c+, e+; M+; N−; S−; s+; P1+; Le(a−b+); K+ k+; Fy(a+b+); Jk(a−b+), and Co(a−b−) Co−3. Family studies revealed that RBCs from her husband, two sisters, and mother all typed as Co(a+b−). Samples from the children were not made available for RBC typing. At prenatal presentation during the second pregnancy, the anti-Co3 was detected. This antibody reacted 1+ in the antiglobulin phase with albumin and ficin methods with anti-IgG (Immucor, Norcross, GA), hereafter referred to as -IgG. The serum titer was 1 (score 9). At 5, 6, and 7 months’ EGA, the antibody was not detected (titer 0). At 8 months the titer was 2 (score 18), and at delivery, the titer showed a moderate increase to 4 (score 21). After delivery the titer decreased to 2 (score 11; Table 2).

Table 1. Primer sequences used for amplification and sequencing of CO (AQP1)

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequence (5’–3’)</th>
<th>Region</th>
<th>Nucleotide position</th>
<th>Exon</th>
<th>Publication</th>
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<td>CoP1</td>
<td>catccctaacatggcatgcagtg</td>
<td>Promoter</td>
<td>−611 to −589</td>
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<td>Joshi et al., 2001</td>
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<tr>
<td>CoP2</td>
<td>aactgctggccaagcttattcc</td>
<td>Intron 1</td>
<td>+291 to +270</td>
<td>1</td>
<td>Joshi et al., 2001</td>
</tr>
<tr>
<td>CoP3</td>
<td>gggctggagtttcattaacacag</td>
<td>Intron 1</td>
<td>−215 to −193</td>
<td>2 to 4</td>
<td>Joshi et al., 2001</td>
</tr>
<tr>
<td>CoP4</td>
<td>cttcacctcctcacaacctacag</td>
<td>3’ UTR</td>
<td>261 to 237</td>
<td>2 to 4</td>
<td>Joshi et al., 2001</td>
</tr>
<tr>
<td>Coln2F</td>
<td>gagcagcgggacactcttg</td>
<td>Intron 2</td>
<td>−169 to −152</td>
<td>Sequencing</td>
<td>This study</td>
</tr>
<tr>
<td>Coln3F</td>
<td>cttaccctgtagcacaacagtt</td>
<td>Intron 3</td>
<td>+29 to +51</td>
<td>Sequencing</td>
<td>This study</td>
</tr>
</tbody>
</table>

UTR = untranslated region.
Novel AQP1 allele associated with Co(a–b–)

Two years later, when two autologous units were donated (see Case Report), the anti-Co3 was 2+ in antiglobulin tests with albumin (Immucor)-IgG and PEG (Sigma, St. Louis, MO)-IgG methods on the first donation and 1+ in albumin-IgG and 2+ in ficin (Sigma)-IgG.

At 4 months’ EGA in the third pregnancy, the anti-Co3 was 3+ in albumin-IgG and 2+ in ficin-IgG. The titer was 16 (score 44). At 6 months’ EGA, the antibody was 2+ in albumin-IgG with a titer of 8 (score 33). At delivery at 8 months’ EGA the albumin-IgG reactivity was 2+ and 3+ in ficin-IgG; a titration was not performed. A sample from child 3 showed a weakly positive (microscopic only) DAT with polyspecific antiglobulin sera (Immucor) only; anti-IgG and anti-C3 (Immucor) tests were negative.

At delivery of child 4, the proband’s sample showed the anti-Co3 to react 1+ by the albumin-IgG method and 1+ in ficin-IgG. A subsequent sample obtained and evaluated more than a year later when she donated an autologous unit was 2+ in albumin-IgG; titers were not performed in either the delivery sample from the fourth pregnancy or the autologous unit.

No additional antibodies were detected in any of the samples tested over the years, and none of the babies required treatment for HDFN.

Molecular Analysis

DNA sequence analysis of CO (AQP1) from the proband revealed she was homozygous for a G nucleotide deletion at position 601 in exon 3 (nt601delG). This deletion is predicted to cause a premature stop (Val201Stop) in the protein and silencing of the antigen (Fig. 1). This deletion was on the CO*A allele, as evidenced by homozygosity for nt 134C/C in exon 1.

To confirm loss of expression of AQP1 protein and a null phenotype, RBC membranes from the proband were immunoblotted with a Co3 antibody. A 25-kDa band, representing the AQP1 protein, was detected in the positive control but was not present in RBCs from the proband (Fig. 3).

<table>
<thead>
<tr>
<th>Table 2. Anti-Co3 reactivity and titers, as available</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pregnancy</td>
</tr>
<tr>
<td>-----------</td>
</tr>
<tr>
<td>2nd</td>
</tr>
<tr>
<td>5 months’ gestation</td>
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<tr>
<td>6 months’ gestation</td>
</tr>
<tr>
<td>7 months’ gestation</td>
</tr>
<tr>
<td>8 months’ gestation</td>
</tr>
<tr>
<td>At delivery</td>
</tr>
<tr>
<td>16 months after delivery</td>
</tr>
<tr>
<td>3rd</td>
</tr>
<tr>
<td>5 months’ gestation</td>
</tr>
<tr>
<td>6 months’ gestation</td>
</tr>
<tr>
<td>8 months’ gestation (delivery)</td>
</tr>
<tr>
<td>4th</td>
</tr>
<tr>
<td>12 months after delivery</td>
</tr>
</tbody>
</table>

AHG = anti-human globulin; NT = not tested.

Fig. 1 DNA sequence electropherogram of AQP1 exon 3. (A) Proband (AQP1 null). (B) Consensus (conventional AQP1). The proband was homozygous for a G nucleotide deletion at position 601, which is predicted to cause a premature stop (Val201Stop) in the protein.

Fig. 2 Inheritance of the CO*1N.06 (nt601delG) allele in the proband family. The proband is indicated with an arrow. The CO*1N.06 allele is designated in gray (■) and CO*1 allele in white (□). Family members that were not tested (NT) are indicated with diagonal lines (\).
Western blot analysis. RBC membranes from the proband (Lane 2) immunoblotted with anti-Co3. The 25-kDa band, representing the AQP1 protein, was detected in the control but absent in the proband, as indicated.

Discussion

An AQP1 null allele with a deletion of G at nt 601 (601delG) in exon 3 was identified in the proband, whose RBCs typed as Co(a−b−), Co−3. The deletion is on a CO*A allele and causes a premature stop at position 201 (Val201Stop). Absence of the AQP1 protein in the RBCs from the proband was confirmed by Western blot. Molecular analysis of family members showed Mendelian inheritance, with the null allele present in the proband’s mother, one sibling, and two children.

The Co(a−b−) phenotype is extremely rare, and individuals with this phenotype can make anti-Co3, which has been associated with HDFN. In this report, the maternal titer was assessed throughout multiple pregnancies and did not exceed 16 during gestation. Child 2 had a positive DAT (1+) with anti-IgG and did not require treatment. Child 3 had a very weak DAT (microscopically positive) reactive with anti-IgG, with no treatment reported. Although autologous units were collected from the proband, no units were transfused. The antibody in this proband was not clinically significant as evidenced by a titer less than 32 and asymptomatic children. Because Co(a−b−) units are rare and anti-Co3 has been reported to cause complications in pregnancy, and no compatible family members were found, autologous units were recommended; however, none of the babies required treatment for HDFN.

While this paper was under review, the same allele was identified in a Gypsy woman who developed an anti-Co3 phenotype and high-titer anti-Co3 associated with mild HDN. Obstet Gynecol 1989;73 (5 Pt 2):870–2.

References

11. Joshi SR, Wagner FF, Vasantha K, Panjwani SR, Flegel WA. An AQP1 null allele in an Indian woman with Co (a−b−) phenotype and high-titer anti-Co3 associated with mild HDN. Transfusion 2001;41:1273–8.

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Routine adsorption procedures to remove autoantibodies from patients' serum often require many hours to perform. This time-consuming process can create significant delays that affect patient care. This study modified the current adsorption method to reduce total adsorption time to 1 hour. A ratio of one part serum to three parts red blood cells (RBCs; 1:3 method) was maintained for all samples. The one part serum was split into three tubes. Each of these three aliquots of serum was mixed with one full part RBCs, creating three adsorbing tubes. All tubes were incubated for 1 hour with periodic mixing. Adsorbed serum from the three tubes was harvested, combined, and tested for reactivity. Fifty-eight samples were evaluated using both the current method and the 1:3 method. Forty-eight (83%) samples successfully adsorbed using both methods. Twenty (34.5%) samples contained underlying alloantibodies. The 1:3 method demonstrated the same antibody specificities and strengths in all 20 samples. Eight samples failed to adsorb by either method. The 1:3 method found previously undetected alloantibodies in three samples. Two samples successfully autoadsorbed but failed to alloadsorb by either method. The 1:3 method proved to be efficient and effective for quick removal of autoantibodies while allowing for the detection of underlying alloantibodies.

**Materials and Methods**

**Samples**

A total of 58 patient samples known to contain warm autoantibodies were obtained at random. Samples were required to have exhibited autoantibody reactivity and to have had either autologous or allogeneic adsorptions performed using current methods. Three types of samples were used for comparison testing: (1) those that successfully autoadsorbed or alloadsorbed and demonstrated no underlying alloantibodies; (2) those that successfully autoadsorbed or alloadsorbed and demonstrated underlying alloantibodies; and (3) those that did not successfully autoadsorb or alloadsorb and required allogeneic adsorption using 20 percent polyethylene glycol (PEG) prepared in-house (Sigma-Aldrich, St. Louis, MO).

**Ficin Treatment of Adsorbing Cells**

Ficin-treated allogeneic RBCs for warm adsorption were selected to match the patient's Rh, K, Kidd, and Ss phenotype. The volume of RBCs was determined accordingly to yield the required volume; a 3-mL aliquot was generally used. RBCs were obtained from designated “adsorbing units.” The current published adsorption procedure\(^1\) (current method) used by reference laboratories and transfusion services can require 4 to 6 hours to complete and is not guaranteed to successfully remove the autoantibodies.

A less time-consuming alternative is needed to expedite the adsorption process and, at the same time, effectively remove the warm autoantibodies. One method would be to increase the RBC-to-serum ratio in an attempt to more effectively remove autoantibodies. Increasing the ratio of RBCs provides more antigen sites to adsorb the autoantibodies; however, this method has been reported to cause dilution of the serum.\(^1\)

This study evaluated a modified, less time-consuming adsorption procedure that could potentially yield results comparable to those produced by the current method. The modified adsorption procedure involved adjusting the initial serum-to-RBC volumes to a 1:3 ratio (1:3 method) and thus making more antigen sites available to adsorb warm autoantibodies.

**Key Words:** autoantibody, alloantibody, autoadsorption, alloadsorption

Red blood cell (RBC) autoantibodies, when present in the serum of a patient, will react with the patient’s RBCs as well as with all normal RBCs. These autoantibodies have the potential of masking the presence of underlying clinically significant alloantibodies. When a patient with warm autoantibodies in the serum is in urgent need of an RBC transfusion, the time-intensive adsorption process to remove autoantibodies can adversely impact patient care. The current published adsorption procedure\(^1\) (current method) used by reference laboratories and transfusion services can require 4 to 6 hours to complete and is not guaranteed to successfully remove the autoantibodies.

A less time-consuming alternative is needed to expedite the adsorption process and, at the same time, effectively remove the warm autoantibodies. One method would be to increase the RBC-to-serum ratio in an attempt to more effectively remove autoantibodies. Increasing the ratio of RBCs provides more antigen sites to adsorb the autoantibodies; however, this method has been reported to cause dilution of the serum.\(^1\)
**Adsorption Using the Current Published Method**

All samples selected for this study had adsorptions performed using the current method\(^1\) (Fig. 1). Equal volumes of patient serum and ficin-treated adsorbing RBCs were mixed and incubated at 37°C for 30 minutes to 1 hour with periodic mixing. The tube was centrifuged for 5 minutes, and the one-time adsorbed serum was harvested. Testing for adsorption effectiveness was performed in the same phases for which neat serum demonstrated reactivity and included the following: low-ionic-strength saline (LISS)-37°C (ImmuAdd, Low Ionic Strength Medium; Immucor, Norcross, GA), LISS-antihuman globulin (AHG), and PEG-AHG. Tubes were incubated at 37°C for 15 minutes for PEG and 20 minutes for LISS-AHG. After washing four times with saline, two drops of anti-IgG (Immucor) were added to each tube, and the tubes were centrifuged and read for agglutination. Testing that showed reactivity was followed with additional adsorptions. Adsorption was repeated by transferring the one-time adsorbed serum to another fresh aliquot of ficin-treated RBCs for a second adsorption. If necessary, a maximum of three total adsorptions were performed.

**Testing of Adsorbed Serum from the 1:3 Method**

The adsorbed serum was tested against screening cells if the original adsorbed patient sample demonstrated no underlying alloantibodies and against the selected cell panel used for the original antibody workup if the original patient sample demonstrated underlying alloantibodies. If alloantibody reactivity was detected with screening cells, a full panel and selected cells were tested to make identification. Testing was performed in the same phases that showed reactivity in the original case and included the following: LISS-37°C, LISS-AHG, and PEG-AHG. The effectiveness of the 1:3 method was then compared with previous results obtained from standard adsorption testing.

**Statistics**

Adsorption results of the 1:3 method were compared with those of the current method. If present, reactivity of each alloantibody in the adsorbed serum was scored for each method using the published scoring system.\(^1\) Data were statistically analyzed using the paired t test. The level of significance was established at a probability value of less than 0.05.

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Results

Results of the 1:3 method showed 48 of 58 samples (83%) successfully adsorbed, matching the current method (Table 1). Of those 48 samples with successful adsorptions, 20 (34.5%) were known from previous testing to contain alloantibodies. Eight samples failed to be fully adsorbed by either method. Three samples (26, 27, and 28) demonstrated underlying alloantibodies (two anti-E, one anti-f) with only the 1:3 method. Two samples (57 and 58), which previously were successfully adsorbed using autologous RBCs, failed to adsorb using allogeneic RBCs with both the current and 1:3 methods on parallel testing. Two samples (30 and 39) contained underlying IgG antibodies of unknown specificity. In the 20 samples known to contain underlying alloantibodies, the 1:3 method demonstrated the same antibody specificities and comparable reaction strengths as the current method (p = 0.82), with one sample (38) showing a stronger reaction by the 1:3 method than by the current method (Table 2). Sample 30 was reactive 1+ LISS-AHG with three of nine panel cells. Sample 39 was reactive 2+ LISS-AHG and PEG-AHG with four of eight panel cells.

Discussion

An important component of pretransfusion testing is to detect clinically significant alloantibodies. Patients with warm autoantibodies in their serum present a unique and challenging problem because the autoantibodies are broadly reactive, reacting with almost all RBCs tested. Warm autoantibodies are the most common cause of autoimmune hemolytic anemia (AIHA), with the incidence of these antibodies increasing with patient age. Although hemolytic transfusion reactions can occur when patients with clinically significant alloantibodies are transfused with RBCs carrying antigens corresponding to the alloantibodies, acute reactions are unlikely when RBC incompatibility is caused by autoantibody alone. Survival of transfused RBCs is generally the same as survival of the patient’s own RBCs, and transfusion can be expected to have significant temporary benefit. Patients with AIHA can have autoantibodies present in their serum. Warm autoantibodies can cause serologic anomalies including spontaneous agglutination that can result in discrepant ABO and Rh testing. More importantly, warm-reactive autoantibodies can mask the presence of clinically significant alloantibodies. Published data indicate that alloantibodies were detected in 209 of 647 serum samples (32%) of patients with AIHA. Undetected alloantibodies may cause increased hemolysis after transfusion, which can be falsely attributed to an increase in the severity of AIHA. Furthermore, although fatalities caused by undetected clinically significant alloantibodies have declined in recent years, the detection of these alloantibodies is still necessary to prevent serious outcomes. When blood transfusion is ordered for a patient with autoantibodies in the serum, specialized serologic testing including adsorption studies, patient phenotyping, and eluate testing are helpful. A knowledge of the patient’s complete phenotype is useful to predict which clinically significant alloantibodies can potentially be present in the patient’s serum.

One of the most important testing procedures for a patient with AIHA, especially if the patient has a history of pregnancy or transfusion, is adsorption testing to remove autoantibody from the patient’s serum and allow for the detection and identification of clinically significant alloantibodies. Adsorption using autologous RBCs is the best procedure to detect clinically significant antibodies. However, autoadsorption should not be performed on samples from patients who have been recently transfused because transfused donor RBCs might adsorb alloantibodies, resulting in a falsely negative test result.

For patients with recent transfusions, the use of allogeneic RBCs is helpful in adsorbing autoantibodies, leaving behind alloantibodies in the adsorbed serum. If the patient’s phenotype is known, one allogeneic adsorbing cell can be selected to match the patient’s phenotype. The selection of cells is made easier by enzyme treating the allogeneic adsorbing cells to destroy the MNS and Duffy antigens. When the patient’s phenotype is unknown, differential adsorption can be performed using group O RBCs of three different Rh phenotypes: R\(_{R_{c}}\), R\(_{R_{c}}\), and rr; one cell should lack the Jk\(^{a}\) antigen, and another should lack the Jk\(^{b}\) antigen. Aliquots from each Rh phenotype are prepared and three separate adsorptions are performed, one with each Rh phenotype. Adsorbed serum from each adsorption can only be used to rule out alloantibodies corresponding to the antigens lacking on each adsorbing RBC phenotype. For example, R\(_{R_{c}}\), Jk\(^{a}\) and not D, E, c, or Jk\(^{b}\).

Adequate testing to detect alloantibodies in the serum of a patient with autoantibodies may take 4 to 6 hours. Adsorption testing using the current method is time consuming and often results in delay of patient transfusion. Also, should routine allogeneic adsorption fail to remove autoantibody reactivity, a PEG-allogeneic adsorption should be performed. PEG-allogeneic adsorption is a faster adsorption method involving...
Table 1. Summary of data from all samples tested

<table>
<thead>
<tr>
<th>Sample</th>
<th>Autologous adsorption</th>
<th>Allogeneic adsorption required</th>
<th>Underlying alloantibodies detected by current method</th>
<th>Underlying alloantibodies detected by 1:3 adsorption method</th>
<th>1:3 method successful at removing autoantibody reactivity?</th>
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<tr>
<td>1</td>
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PEG = polyethylene glycol.
adsorption with one part RBCs, one part PEG, and one part serum for 15 minutes up to a total of three adsorptions. After adsorption, four to six drops of PEG-adsorbed serum are used to test with each panel cell originally reactive with neat serum. Additional enhancement media are not needed because of the PEG present in the PEG-adsorbed serum. Although PEG adsorption is a faster procedure than routine allogeneic adsorption, there is the risk that weak alloantibodies will not be detected. Knowing sooner whether PEG adsorption is necessary aids in reducing the overall turnaround time of making blood available for transfusion.

This study showed that the 1:3 method gave results comparable to those of the current adsorption method, and in much less time, for those samples that originally required more than one adsorption. Of the 58 serum samples selected at random for this study, 48 were successfully adsorbed using both the current and 1:3 methods. Of these 48 samples, 20 (34.5%) contained underlying alloantibodies, which is consistent with the average of 32 percent from published data. In all 20 samples with underlying alloantibodies, the 1:3 method demonstrated the same antibody specificities and reaction strengths as the current method, with one sample yielding stronger alloantibody reactivity in the 1:3 method. Eight samples that failed to be adsorbed by the current method also failed with the 1:3 method. The modified 1:3 method detected underlying alloantibodies in three samples that were not detected using the current method. Two samples that successfully adsorbed in previous testing using autologous RBCs failed to adsorb by the 1:3 method using allogeneic adsorbing RBCs. Further parallel adsorption testing using two separate allogeneic adsorbing RBCs showed both samples failed to adsorb using both the current and 1:3 methods. An explanation could not be found in either of the two samples that successfully autoadsorbed, but failed to alloadsorb by routine methods, as to why only autologous adsorption could remove autoantibody reactivity.

Other studies reported that reductions in adsorption incubation times to as little as 10 minutes are equally effective as currently accepted standard methods. Possible future studies could combine this 1:3 method with a shortened incubation time to evaluate whether autoantibodies could still be effectively removed without adverse impact on the final results.

Summary

Standard adsorptions can require 4 to 6 hours; the 1:3 method required approximately 1 to 1.5 hours for the entire adsorption process. In conclusion, this study showed the 1:3 method of using one patient’s serum to three parts RBCs to be time efficient as well as effective for quick removal of autoantibodies while allowing for the detection of underlying alloantibodies.

References


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Major non-ABO incompatibility caused by anti-Jk\(^a\) in a patient before allogeneic hematopoietic stem cell transplantation

M.Y. Kim, P. Chaudhary, I.A. Shulman, and V. Pullarkat

A 49-year-old white man with blood group AB, D+ was found to have alloanti-Jk\(^a\) and -K when he developed a delayed hemolytic transfusion reaction before allogeneic hematopoietic stem cell transplant (HSCT). Given that his stem cell donor was blood group O, D+, Jk(a+), K–, rituximab was added to his conditioning regimen of fludarabine and melphalan to prevent hemolysis of engrafting Jk(a+) donor red blood cells. The patient proceeded to receive a peripheral blood stem cell transplant from a matched unrelated donor with no adverse events. To our knowledge, this is the first case of successful management of major non-ABO incompatibility caused by anti-Jk\(^a\) in a patient receiving an allogeneic HSCT reported in the literature. *Immunohematology* 2013;29: 11–14.

**Key Words:** anti-Jk\(^a\), major incompatibility, hematopoietic stem cell transplantation

ABO mismatch is well characterized in the setting of hematopoietic stem cell transplant (HSCT). Because of the disparate genetic locations of HLA and ABO genes, ABO mismatch occurs in 30 to 50 percent of transplants.\(^1\) Red blood cell (RBC)–incompatible transplantation does not seem to have an adverse effect on transplant outcomes, such as engraftment, graft-versus-host disease (GVHD), relapse, or survival.\(^2\) However, it does carry the risk of hemolytic transfusion reactions (HTR), and it must be managed appropriately with interventions such as graft processing and proper blood component support.

RBC incompatibility can be classified into two categories. Major incompatibility is when the recipient has antibodies directed against donor RBC antigens. Minor incompatibility is defined as a donor having antibodies directed against recipient RBC antigens. Bidirectional incompatibility, usually in a group A donor with a group B recipient or vice versa, is when there is both major and minor incompatibility. Major incompatibility carries the risk of acute HTR and also delayed RBC recovery after transplant. Minor incompatibility can result in "passenger lymphocyte syndrome," in which the donor B lymphocytes produce antibodies against recipient RBCs that cause a delayed hemolytic transfusion reaction 7 to 12 days after transplantation.

Patients undergoing HSCT require frequent RBC transfusions owing to both the underlying disease and treatment with chemotherapy or radiation.\(^3\) Frequent transfusions predispose patients to developing alloantibodies to non-ABO RBC antigens. RBC alloantibodies may become undetectable over time and with restimulation can cause a delayed HTR if their past existence is not known before transfusion.\(^4\) Non-ABO antigens such as those in the Rh, Kell, and Kidd blood group systems have been implicated as targets for passenger lymphocyte syndrome after transplant.\(^5–8\)

We present a patient who developed an unusual form of bidirectional incompatibility in the form of minor ABO incompatibility, being a group AB recipient with a group O donor, and major non-ABO incompatibility, with preformed anti-Jk\(^a\) directed against the donor’s Jk(a+) RBCs.

**Case Report**

A 49-year-old man was diagnosed with primary myelofibrosis in 2007. He was treated with hydroxyurea until May 2011, when he developed worsening anemia and thrombocytopenia that did not improve after hydroxyurea was discontinued. Bone marrow biopsy revealed advanced reticulin fibrosis. The patient had massive splenomegaly of 26 cm. His blood group was AB, D+, and he had a negative screening test for RBC alloantibodies. He had received 38 units of group AB, D+ RBC transfusions over a period of 8 months. Because his antibody screening test was negative, neither the patient nor any of the 38 RBC units were phenotyped for antigens beyond the standard A, B, and D. At this point it was decided that the patient needed an allogeneic HSCT.

The patient was admitted to our hospital for a matched unrelated donor HSCT. He received reduced-intensity conditioning with fludarabine 25 mg/m\(^2\)/day given intravenously from Day −9 to Day −5 and melphalan 140 mg/m\(^2\) given intravenously on Day −4. GVHD prophylaxis was initiated with tacrolimus 0.02 mg/kg/day continuous infusion starting on Day −3 and sirolimus 12 mg on Day −3 and 4 mg
Peripheral blood stem cells were mobilized from the donor with granulocyte colony-stimulating factor, and $8 \times 10^6$ CD34+ cells/kg were infused into patient. Patient and donor were 10 of 10 HLA matched, with minor ABO incompatibility as the donor was group O, D+.

On admission to our hospital, the patient’s screening test for unexpected RBC antibodies was positive, but no specific antibody could be identified. Because of the positive antibody screen and because the prospective stem cell donor was group O, RBC units selected for transfusion were group O, D+ and crossmatched using an indirect antiglobulin test. On pretransplant Day –8, the patient was transfused with one RBC unit as his hemoglobin was 7.3 g/dL. On pretransplant Day –6, the patient received an additional two units of RBCs as his hemoglobin was again low, 6.8 g/dL. On pretransplant Day –5, the patient was found to have an anti-Jk$. On further review it was found that one of the RBC units transfused on Day –6 was Jk(a+). At this point the patient’s direct antiglobulin test (DAT) was positive with IgG and C3 coating his RBCs. An eluate prepared from his RBCs contained anti-Jk$. On Day –4, the patient’s hemoglobin dropped from 8.4 g/dL to 6 g/dL, his lactate dehydrogenase (LDH) rose to 942 IU/L, and his total bilirubin rose to 2.8 mg/dL, all consistent with a delayed HTR (Fig. 1). He required five units of RBCs to maintain an adequate hemoglobin level over the next 3 days. Given that the donor was Jk(a+), one dose of rituximab 375 mg/m$^2$ intravenously was added on Day –3 to prevent hemolysis after donor cell engraftment, as well as to reduce likelihood of passenger lymphocyte engraftment given the minor ABO incompatibility.

On pretransplant Day –2, the patient was also found to have an anti-K. No RBC units transfused during this admission were positive for K and the donor was K–, so this was not investigated further.

**Materials and Methods**

The patient’s ABO/D status was verified using monoclonal anti-A, -B, and -D reagents (BioClone, Ortho Clinical Diagnostics, Inc., Raritan, NJ). Initial screens for RBC antibodies were performed using the gel test (MTS Anti-IgG Card, Micro Typing Systems, Inc., Pompano Beach, FL). Monoclonal anti-Jk$^a$ typing reagent (BioClone) was used to detect circulating Jk(a+) RBCs. Monoclonal anti-IgG,

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**Fig. 1** Patient’s hemoglobin (Hb), lactate dehydrogenase (LDH), and bilirubin (T-bili) trend during transplant (occurring on Day 0). Each arrow at the top of the graph represents one transfused unit of red blood cells (RBCs). Solid arrows represent group O, D+, Jk(a–) RBC units; dashed arrow represents the one group O, D+, Jk(a+) RBC unit the patient received on Day –6. Anti-Jk$^a$ was detected on Day –5.
-C3d, polyspecific AHG (BioClone) were used to perform the DAT. Monoclonal Anti-AB containing the ES4 clone (anti-A,B Murine Monoclonal Blend Series 1, Immucor Gamma, Norcross, GA) was used to type the patient’s ABO status after transplant. Both the gel test (MTS, Anti-IgG card, Ortho) and automated solid-phase capture assay (Galileo Echo; Immucor Gamma) were used to serially monitor the patient’s anti-Jk\(^a\).

**Results**

By the day of transplantation, the patient’s hemoglobin had stabilized, and LDH and bilirubin were back to baseline. After transplantation, the patient received daily RBC transfusions with group O, D+, Jk(a–), K– RBCs from Day +4 to Day +10; during this time there was no increase in markers of hemolysis, and the DAT remained negative. The patient achieved neutrophil engraftment on Day +11. On the same day, the patient first typed macroscopically as group O, D+ Jk(a–), a reflection of the 11 units of matched RBCs transfused during the preceding 2 weeks. On Day +21, the patient typed as O+, Jk(a+), indicating successful RBC reconstitution with donor erythropoiesis. Of note, with standard anti-A and anti-B typing, the patient forward typed as group O; however, with anti-A/B from an ES4 clone, the patient continued to show 1+ reactivity.

The patient’s anti-Jk\(^a\) was initially detected at a titer of 2 on Day –5. On serial monitoring, the titer decreased to 1 on Day +14 and became undetectable using gel-column assay (MTS Anti-IgG card, Ortho) on Day +17. Using solid-phase adherence assay (Capture, Immucor) the antibody was still detected until Day +40, converted to negative on Day +49, and remained so thereafter. Despite the persistence of detectable antibody, the patient required only two additional units of RBCs, one each on Day +24 and Day +40, and his posttransplant course remained uneventful otherwise.

**Discussion**

In this case, we report a patient who developed alloantibodies to both Jk\(^a\) and K before allogeneic HSCT. Neither of these antibodies was detectable during the time leading up to his admission for HSCT, during which period his transfusion support was provided outside of our institution. Although we do not know the Jk\(^a\) status of his prior transfusions, the prevalence of Jk\(^a\) and K suggest that at least 15 of the 20 units he received in the 3 months before admission would have been Jk(a+), and 2 units would have been K+. Unfortunately, the patient had almost completed his conditioning regimen for HSCT when the anti-Jk\(^a\) and -K were identified. This example of anti-Jk\(^a\) detected in our patient was clinically significant as evidenced by its ability to cause a delayed HTR, necessitating aggressive RBC transfusion support for a short time before transplant. There is scant literature available about the management of this type of situation.

The paucity of data regarding this issue likely is because patients undergoing HSCT have a low incidence of alloantibodies relative to the number of RBC transfusions they receive.\(^9\) The lack of alloimmunization is thought to be related to the intense chemotherapy given to patients for their underlying hematologic disease before transplant. Patients like ours constitute a minority of HSCT candidates who did not receive chemotherapy other than hydroxyurea before transplant, and this may have increased his likelihood of developing RBC alloantibodies.

Even when patients have alloantibodies, the traditional conditioning regimen before HSCT with chemotherapy or radiation will usually prevent these antibodies from being able to act effectively against donor RBCs. In one study, of 14 patients who were found to have alloantibodies before transplant, all but one no longer had detectable antibodies after transplant,\(^9\) supporting the hypothesis that RBC alloantibodies can be eradicated in patients with HSCT.

However, as the indications for HSCT expand to include nonmalignant diseases such as thalassemia and sickle cell disease, and the use of reduced-intensity conditioning regimens continues to increase, non-ABO incompatibility issues may become a more prevalent problem in the future. For example, Borge et al.\(^10\) reported a patient with sickle cell disease who also had anti-Jk\(^a\) and received HSCT from a Jk(a+) donor. In this case the recipient had delayed RBC engraftment that persisted for more than 180 days after transplant. A nonmyeloablative conditioning regimen was used, consisting of alemtuzumab 1 mg/kg and a single dose of total body irradiation 300 cGy, while oral sirolimus was used for prevention of GVHD.\(^11\)

Although we also used a reduced-intensity conditioning regimen, we believe that in our patient the immunosuppressive agents, fludarabine and rituximab, were key in preventing potential problems. Fludarabine, a purine nucleoside analog, is a highly immunosuppressive agent that causes myelo-suppression and prolonged reduction of CD4+ lymphocytes.\(^12\) Rituximab is an anti-CD20 monoclonal antibody that selectively depletes circulating B cells, thus preventing antibody production.\(^13\) These two agents in combination effectively target all the immune cells required to generate an HTR. Eventually, with achievement of complete donor chimerism,
the donor immune cells would be expected to completely eliminate any residual alloantibody-producing cells.

For ABO-incompatible HSCT, delayed RBC engraftment has been associated with the length of time for the isohemagglutinin titers to decrease to clinically insignificant levels (1+ or lower in strength). In our case, the initial anti-Jk<sup>a</sup> titer was 2 and became 1+ on Day +14, while donor erythropoiesis was established on Day +21. Thus, it may be useful to monitor antibody titers in the non-ABO–incompatible HSCT setting as an early indicator of whether successful donor erythropoiesis may be achieved. Whether high initial titers or persistent antibody detection warrants further intervention has yet to be determined.

Of note, the increased sensitivity of laboratory methods used in the detection of RBC antigens and antibodies may also cause RBC compatibility issues in HSCT to become more prevalent in the future. For instance, the anti-Jk<sup>a</sup> in our patient could be detected on solid-phase capture assay up to 32 days after the gel-column assay became negative. Also, although the patient first typed as blood group O on Day +11, he continued to have detectable A and B on his RBCs when using the more sensitive assay with the ES4 clone.

In conclusion, we present the case of a patient with minor ABO and major non-ABO mismatch who successfully received matched unrelated donor HSCT. We believe that major non-ABO mismatch is an underreported phenomenon that usually does not lead to clinically significant consequences, and clinicians should not be discouraged from using a major non-ABO–incompatible donor if such an incompatibility is discovered before HSCT. However, close monitoring of the recipients with markers for serum hemolysis should be performed during HSCT because severe hemolysis can occur in such situations. Monitoring antibody titers may also be helpful in predicting its clinical relevance. Depending on the level of concern, rituximab may be an additional strategy to use in this situation to avoid hemolysis and ensure successful RBC engraftment. We feel that this is a clinical situation that may become more common in the future given changing patterns of HSCT and the increased sensitivity of assays used to detect RBC antigens and antibodies.

References


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A case of autoimmune hemolytic anemia with anti-D specificity in a 1-year-old child

R.S. Bercovitz, M. Macy, and D.R. Ambruso

Although antibodies to antigens in the Rh blood group system are common causes of warm autoimmune hemolytic anemia, specificity for only the D antigen is rare in autoimmune hemolysis in pediatric patients. This case reports an anti-D associated with severe hemolytic anemia (Hb = 2.1 g/dL) in a previously healthy 14-month-old child who presented with a 3-day history of low-grade fevers and vomiting. Because of his severe anemia, on admission to the hospital he was found to have altered mental status, metabolic acidosis, abnormal liver function tests, and a severe coagulopathy. He was successfully resuscitated with uncrossmatched units of group O, D– blood, and after corticosteroid therapy he had complete resolution of his anti-D-mediated hemolysis. *Immunohematology* 2013;29:15-18.

Key Words: autoimmune hemolytic anemia, pediatrics, anti-D

Autoimmune hemolytic anemia (AIHA) is the pathological destruction of red blood cells (RBCs) by antibodies produced against self-erythrocyte surface antigens. Its prevalence is estimated to be approximately 1 to 3 per 100,000 per year, although it may be lower in pediatric patients. Warm AIHA is usually caused by immunoglobulin G (IgG) antibodies that bind to RBC antigens and result in erythrophagocytosis by splenic macrophages or hepatic Kupffer cells. In many cases, antigen specificity cannot be determined, or patients express pan-reactivity across antigen groups. However, there have been reports of specificity to as many as 50 RBC antigens with anti-e being one of the most common specificities cited in reviews.4–6

AIHA can be either a primary or a secondary disease, usually as a result of an underlying autoimmune disease, primary immunodeficiency, or lymphoid malignancy; it can present in a known primary process or as part of its initial presentation.4,7,8 In adult patients primary AIHA represents approximately 60 percent of cases.9 In case series of pediatric patients, the proportion of patients with primary AIHA has ranged from 7 to 64 percent.5,5,10 This case of AIHA is unusual because of the D specificity of the autoantibody and its occurrence in a 14-month-old child without an underlying immune or autoimmune disorder and with no long-term sequelae.

A previously healthy 14-month-old white male born after a term pregnancy without perinatal problems and with no prior history of blood transfusion presented to the emergency department with lethargy and jaundice. He had a history of low-grade fevers, vomiting, and fatigue for 3 days before presentation. On the day of admission he was noted to have occasional episodes of shallow breathing with decreased responsiveness. His vital signs showed he was tachycardic, normotensive, and not hypoxic. He was noted to be pale, jaundiced, and responsive to painful stimulus only. In addition, he was found to have an intermittent gallop and hepatosplenomegaly.

His initial blood work showed he was severely anemic with a hemoglobin (Hb) of 2.1 g/dL, hematocrit (Hct) of 7.1 percent, and an elevated reticulocyte count of 32 percent. His white blood cell count was elevated, and his platelet count was normal. The pertinent laboratory evaluations are summarized in Table 1. In addition to his severe anemia, the patient had a bilirubin that was greater than 3 times the upper limit of normal and a lactate dehydrogenase, which is a marker for rapid cell turnover, that was almost 6 times the upper limit of normal. The results were consistent with the diagnosis of an acute hemolytic anemia.

Further laboratory testing demonstrated significant end-organ ischemia secondary to his severe anemia. He was acidotic on admission, with a pH of 7.19. He had evidence of prerenal insufficiency and hepatic dysfunction with elevated hepatocellular enzymes. Although he did not have any clinical signs of bleeding, he had a prolonged prothrombin time, but a normal partial thromboplastin time. Further evaluation of coagulation factors demonstrated a deficiency in factors II, V, and VII, an elevated factor VIII, and normal fibrinogen. His D dimer was 1390 ng/mL. Although there was evidence of activation of coagulation, the patient did not have severe consumption, and his coagulopathy was most likely attributable to decreased hepatic synthesis. Vitamin K deficiency could not be documented.
He was resuscitated with both crystalloid fluids and emergency units of unmatched, group O, D– packed RBCs. After this resuscitation the patient’s mental status and cardiovascular status improved. His renal and liver function tests improved, and he had prompt resolution of his metabolic acidosis. Further testing showed that the patient was group B, D+ with warm-reacting autoantibodies. His direct antiglobulin test (DAT) was 2+ positive for IgG, and an eluate from the cells demonstrated anti-D specificity and no reactivity with D+ LW– RBCs. Extended Rh phenotype by serology indicated that the patient was D+, C–/c+, E+/e– (likely R_2R_2); however DNA testing revealed that the patient’s genotype was D heterozygote, C–/c+, and E+/e+ (likely R_2R_2). There was no evidence of anti-C/c or anti-E/e alloantibodies or autoantibodies. Sequencing of his RHD gene showed he was negative for the RHD-inactivating pseudogene and had none of the 18 most common partial D genotypes. The discrepancy between his positive e genotype and negative phenotype for e is likely caused by an altered RHCE gene, although complete sequencing could not be performed.

After his initial resuscitation, the patient’s hemoglobin remained stable with no additional evidence of hemolysis, and he did not require any additional RBC transfusions. On the day of admission, he was started on a 10-day course of prednisone (2 mg/kg per day) and was successfully tapered off the medication without recrudescence of his hemolysis. Infectious disease testing was performed, including a respiratory virus direct stain for adenovirus, influenza A and B, parainfluenza 1 through 3, and respiratory syncytial virus, which was negative. There was no evidence of current or prior infection with Epstein-Barr virus. The patient had no underlying conditions such as another autoimmune disorder (negative antinuclear antibody), immunodeficiency (normal serum immunoglobulins), or malignancy, making this a primary AIHA.

Samples from the patient exhibited a weakly positive DAT for 2 to 3 months after his initial presentation. Subsequently, the DAT became negative, and he had complete resolution of his hemolysis 1 year after his initial presentation without evidence of any autoimmune or immune disorders.

### Table 1. Selected abnormal laboratory values in this patient consistent with a brisk hemolytic process and end-organ ischemia caused by severe anemia

<table>
<thead>
<tr>
<th>Laboratory test</th>
<th>Patient’s results</th>
<th>Normal range</th>
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<td>Complete blood count</td>
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<tr>
<td>White blood cells (WBC)</td>
<td>39.4 × 10^3/µL</td>
<td>5–13 × 10^3/µL</td>
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<td>Hemoglobin (Hb)</td>
<td>2.1 g/dL</td>
<td>9.5–14 g/dL</td>
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<td>Hematocrit (Hct)</td>
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<td>Platelet count</td>
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<td>150–500 × 10^3/µL</td>
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<td>Reticulocyte count</td>
<td>32%</td>
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<td>Venous blood gas</td>
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<td>pH</td>
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<td>7.32–7.42</td>
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<td>PCO₂</td>
<td>19 mm Hg</td>
<td>40–50 mm Hg</td>
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<td>Bicarbonate (HCO₃)</td>
<td>7 mEq/L</td>
<td>22–25 mEq/L</td>
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<td>Glucose</td>
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<td>60–105 mg/dL</td>
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<td>Blood urea nitrogen (BUN)</td>
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<td>Creatinine</td>
<td>0.7 mg/dL</td>
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<td>Lactate dehydrogenase (LDH)</td>
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<td>Liver function tests</td>
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<td>Bilirubin (total)</td>
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<td>Alanine transaminase (ALT, SGPT)</td>
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<td>Coagulation tests</td>
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<td>Prothrombin time (PT)</td>
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<td>Partial thromboplastin time (PTT)</td>
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<tr>
<td>Factor II</td>
<td>38%</td>
<td>50–150%</td>
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<td>PIVKA II</td>
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<td>Factor V</td>
<td>14%</td>
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<td>Factor VIII</td>
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<td>Fibrinogen</td>
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<td>D dimer</td>
<td>1390 ng/mL</td>
<td>&gt;255 ng/mL</td>
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PIVKA II = protein-induced by vitamin K absence or antagonist II.

Discussion

AIHA is caused by antibodies to a specific antigen on the patient’s own erythrocytes, resulting in either intravascular or extravascular hemolysis. Warm AIHA is caused by IgG antibodies and results in antibody-mediated erythropagocytosis by splenic macrophages. Cold AIHA is caused by IgM antibodies and results in intravascular hemolysis secondary to complement fixation on the RBC surface. The thermal amplitude of the antibodies determines their clinical significance; cold agglutinins that are reactive at temperatures lower than body temperature are generally of little clinical significance. Bifasic IgG antibodies that bind RBCs at colder temperatures and then fix complement in warmer temperatures cause paroxysmal cold hemoglobinuria (PCH).
The incidence of both warm and cold AIHA increases with patient age. But in pediatric patients, the highest incidence of cold AIHA, including cold agglutinin syndrome and PCH, is in patients younger than the age of 4, likely because of their association with common childhood infections such as viral respiratory infections and *Mycoplasma pneumoniae*. In most case series, warm AIHA constitutes about 60% of the cases in pediatric patients. Some series report that primary AIHA is more common, whereas others demonstrate that secondary AIHA is more common in pediatric patients.

One of the largest series showed that the majority of cases of AIHA are caused by warm antibodies, 64% percent, versus 26% attributable to cold antibody and 10% percent attributable to mixed antibodies (n = 100). This series also demonstrated that approximately half of the patients (54%) had an underlying disease process such as autoimmune disease, idiopathic thrombocytopenia, neoplasia, or hemoglobinopathy, whereas the remaining 46% of patients had primary (idiopathic) AIHA. The most common autoimmune disorders associated with AIHA include lupus, Evan’s syndrome, autoimmune lymphoproliferative syndrome (ALPS), and other immunodeficiencies. The majority of patients with warm antibody disease, 59% (38/64), had primary AIHA, similar to a series of 26 children in India that showed that 65% had primary AIHA. Children with primary AIHA are more likely than adults to have a self-resolving, relatively short course (less than 6 months). Patients who present at younger than 2 years and older than 12 years are at risk for a chronic course.

The discrepancy between the patient’s e negative phenotype and e positive genotype likely represents an altered RHCE gene. This altered gene may place the patient at a higher risk of developing alloantibodies to the e antigen; however, it should not play a role in the development of autoantibodies to D. D is the most immunogenic antigen when development of alloantibodies occurs after an exposure; however, it is not commonly associated with autoantibody development. Antibodies against antigens in the Rh system, such as anti-e, anti-E, and anti-c, are most commonly implicated in warm AIHA. Patients frequently have multiple anti-Rh antibodies or panreactive Rh antibodies, but having only anti-D is rare in AIHA. There was an additional case report of IgM anti-D in the setting of non-Hodgkin lymphoma. To date, there is only one case of primary AIHA caused by anti-D in an adult patient. To our knowledge, the case presented here is a unique case of primary AIHA with an IgG antibody toward the D antigen in a pediatric patient.

### Acknowledgments

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### References


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An update on the GLOB blood group system and collection

Å. Hellberg, J.S. Westman, and M.L. Olsson

The P blood group antigen of the GLOB system is a glycolipid structure, also known as globoside, on the red blood cells (RBCs) of almost all individuals worldwide. The P antigen is intimately related to the P\(^k\) and NOR antigens discussed in the review about the P1PK blood group system. Naturally occurring anti-P is present in the serum of individuals with the rare globoside-deficient phenotypes p, P\(^1\)k, and P\(^2\)k and has been implicated in hemolytic transfusion reactions as well as unfavorable outcomes of pregnancy. The molecular genetic basis of globoside deficiency is absence of functional P synthase as a result of mutations at the \(B3GALNT1\) locus. Other related glycolipid structures, the LKE and PX2 antigens, remain in the GLOB blood group collection pending further evidence about the genes and gene products responsible for their synthesis. *Immunohematology* 2013;29:19–24.

**History**

Reading the early literature about what are currently known as the P1PK and GLOB blood group systems is a bit complicated because of evolving name changes based on increasing knowledge that has improved previously drawn conclusions. The P antigen is also known as globoside, a name given because it was discovered and characterized first on red blood cells (RBCs) (*globule rouge* is French for red blood cell). The antibody now referred to as anti-P1 was originally called anti-P and was initially recognized in 1955 as a component of anti-Tj\(^a\) (now designated anti-PP1P\(^k\)), the mix of naturally occurring antibodies in sera of people with the p phenotype.\(^1\) The first globoside-deficient individual described had the rare P\(^1\)k phenotype and was reported in 1959 by Matson et al.\(^2\) However, the first paper highlighting the relationship between the P1, P\(^k\), and P antigens, as well as determining the biochemical structure of these glycolipids, was published by Naiki et al.\(^3\) 15 years later.

**Terminology and Nomenclature**

The P antigen is so far the only member of the GLOB blood group system acknowledged by the International Society of Blood Transfusion (ISBT) Working Party for Red Cell Immunogenetics and Blood Group Terminology as system number 028. The antigen was first assigned to the P blood group system (as antigen no. 003002), which is the former name of what is now known as the P1PK system (ISBT no. 003), today housing the P1, P\(^k\), and NOR antigens. Thereafter, P was moved to the GLOB blood group collection (ISBT no. 209, antigen no. 209001) when it was clear that P1 and P were only distant relatives. Finally, it was promoted to form a blood group system of its own when the molecular genetic basis for P antigen synthesis was established in 2002 (antigen no. 028001).\(^4\) Other names sometimes used instead of P, especially in the biochemical and glycobiological literature, include globoside, globotetraosylceramide, and Gb4. It should be noted that P was the name formerly used for what is now known as the P1 antigen. The LKE antigen remains in the GLOB collection (ISBT no. 209), together with the newly added PX2 antigen.\(^5\) The current terminology of the GLOB blood group system and collection is summarized in Table 1.

### Table 1. The GLOB blood group system and collection*

<table>
<thead>
<tr>
<th>Antigen</th>
<th>ISBT system no.</th>
<th>ISBT collection no.</th>
<th>ISBT antigen no.</th>
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<td>P</td>
<td>GLOB 028</td>
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<td>028001</td>
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<tr>
<td>LKE</td>
<td>GLOB 209</td>
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<td>209003</td>
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<tr>
<td>PX2</td>
<td>GLOB 209</td>
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<td>209004</td>
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*2090001 and 2090002 are obsolete (previously used for P and P\(^k\)).

### Molecular Genetic Basis of P Antigen

A gene, first cloned in 1998 as a member of the 3-β-galactosyltransferase family\(^6\) but later shown to be a 3-β-N-acetylglactosaminyltransferase, was suggested as the globoside (Gb4, P) synthase.\(^7\) This gene (\(B3GALNT1\), formerly known as \(B3GALT3\)) is located on the long arm of chromosome 3 (3q26.1) and has at least five exons with the entire coding region in the last exon (Fig. 1). The gene encodes the enzyme that synthesizes the P antigen. So far, 12 mutations have been found to abolish P synthase activity (Table 2).\(^4\) Only one noncritical polymorphism has been described in the exons of this gene, in contrast to the P\(^k\) synthase gene (\(A4GALT\)), which varies more in the population.
The P antigen is present on RBCs of all individuals except those with the rare phenotypes p, P<sub>1</sub><sup>k</sup>, or P<sub>2</sub><sup>k</sup>. The P<sub>1</sub><sup>k</sup> phenotype lacks the P antigen on the cell surface, whereas the P<sub>2</sub><sup>k</sup> phenotype lacks both the P and P<sub>1</sub> antigens. The p phenotype, on the other hand, lacks P<sub>1</sub><sup>k</sup>, P, and P<sub>1</sub> antigens. Additional phenotypes might exist as Kundu et al. described individuals with either a weak P or weak P<sub>1</sub><sup>k</sup> antigen, but the genetic basis of this is not known and such individuals appear to be rare.

The P<sub>1</sub><sup>k</sup> and P<sub>2</sub><sup>k</sup> phenotypes are even rarer than the p phenotype but appear to be more common in Japan. The first individual described with the P<sub>1</sub><sup>k</sup> phenotype was of Finnish origin, and it also appears that Finland has a higher prevalence of the rare P<sub>1</sub><sup>k</sup> and P<sub>2</sub><sup>k</sup> phenotypes than do other populations.

The antigen is well developed at birth and is the most abundant neutral glycolipid in the RBC membrane with approximately 15 × 10<sup>6</sup> antigens per cell, probably the highest antigen site density for any blood group antigen. None of the enzymes or chemicals used to treat test RBCs for antigen modification can abolish its expression, but many, including papain and trypsin, markedly enhance it. RBCs from P<sub>1</sub><sup>k</sup> individuals express more P<sub>1</sub><sup>k</sup> antigen compared with P<sub>2</sub><sup>k</sup> individuals, but the amount of P antigen is similar for both phenotypes.

However, because the P<sub>1</sub><sup>k</sup> antigen constitutes the precursor for P synthase, it is possible that the P antigen site density is somewhat lower on P<sub>2</sub><sup>k</sup> individuals, but substantial interindividual variation exists.

Naturally occurring antibodies of IgM or IgG classes are formed when the P antigen is missing. In analogy with ABO antibodies, anti-P can cause hemolytic transfusion reactions of the acute intravascular type, although no clinically significant hemolytic disease of the fetus and newborn has been reported. Nevertheless, early spontaneous abortions occur with a higher frequency among women with p and P<sub>1</sub><sup>k</sup> or P<sub>2</sub><sup>k</sup> phenotype; this is a phenomenon most likely attributable to the IgG component of anti-P attacking certain cells in the placenta, where globoside

### Table 2. A summary of all mutations found to date in the B3GALNT1 gene<sup>4,8,9</sup>

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<tr>
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<td>Change</td>
<td>stop</td>
<td>fs</td>
<td>fs</td>
<td>stop</td>
<td>G</td>
<td>stop</td>
<td>fs</td>
<td>fs</td>
<td>S</td>
<td>A</td>
<td>R</td>
<td>stop</td>
</tr>
</tbody>
</table>

nt = nucleotide, Acc. no. = accession number, aa = amino acid, fs = frameshift.
*Additional mutation present in allele 376G>A (D1'26N).

### Antigens and Antibodies in the System

The P antigen is present on RBCs of all individuals except ones with the rare phenotypes p, P<sub>1</sub><sup>k</sup>, or P<sub>2</sub><sup>k</sup>. The P<sub>1</sub><sup>k</sup> phenotype lacks the P antigen on the cell surface, whereas the P<sub>2</sub><sup>k</sup> phenotype lacks both the P and P1 antigens. The p phenotype, on the other hand, lacks P<sub>1</sub><sup>k</sup>, P, and P1 antigens. Additional phenotypes might exist as Kundu et al. described individuals with either a weak P or weak P<sub>1</sub><sup>k</sup> antigen, but the genetic basis of this is not known and such individuals appear to be rare.

The P<sub>1</sub><sup>k</sup> and P<sub>2</sub><sup>k</sup> phenotypes are even rarer than the p phenotype but appear to be more common in Japan. The first individual described with the P<sub>1</sub> phenotype was of Finnish origin, and it also appears that Finland has a higher prevalence of the rare P<sub>1</sub><sup>k</sup> and P<sub>2</sub><sup>k</sup> phenotypes than do other populations.

The antigen is well developed at birth and is the most abundant neutral glycolipid in the RBC membrane with approximately 15 × 10<sup>6</sup> antigens per cell, probably the highest antigen site density for any blood group antigen. None of the enzymes or chemicals used to treat test RBCs for antigen modification can abolish its expression, but many, including papain and trypsin, markedly enhance it. RBCs from P<sub>1</sub> individuals express more P<sub>1</sub> antigen compared with P<sub>2</sub> individuals, but the amount of P antigen is similar for both phenotypes. However, because the P<sub>1</sub> antigen constitutes the precursor for P synthase, it is possible that the P antigen site density is somewhat lower on P<sub>2</sub> individuals, but substantial interindividual variation exists.

Naturally occurring antibodies of IgM or IgG classes are formed when the P antigen is missing. In analogy with ABO antibodies, anti-P can cause hemolytic transfusion reactions of the acute intravascular type, although no clinically significant hemolytic disease of the fetus and newborn has been reported. Nevertheless, early spontaneous abortions occur with a higher frequency among women with p and P<sub>1</sub><sup>k</sup> or P<sub>2</sub><sup>k</sup> phenotype; this is a phenomenon most likely attributable to the IgG component of anti-P attacking certain cells in the placenta, where globoside
is highly expressed.\textsuperscript{16,17} Plasmapheresis to reduce the maternal antibody titer has been successfully used in selected cases.\textsuperscript{18} Autoanti-P can be formed after viral infections (see Disease Associations).

**Biochemistry**

P antigen belongs to the globoseries of glycolipids. Its structure was first elucidated by Naiki et al., and it was later shown by Yang et al. that glycolipids are the sole carriers of P antigen on RBCs.\textsuperscript{3,19} The P antigen is made with the addition of N-acetylglactosamine (GalNAc) by a 3-β-N-acetylgalactosaminyltransferase (β3GalNAc-T1 classified as EC 2.4.1.79 but also known as P or Gb4 synthase) using P\textsuperscript{k} (Gb3) as a precursor (Fig. 2). This enzyme is a type II transmembrane glycoprotein with 331 amino acids and five potential N-glycosylation sites. Similar to other glycosyltransferases, it resides in the Golgi apparatus, where it is part of the glycosylation machinery of the cell. The crystal structure of this enzyme has not yet been solved, and regulation of its transcription is not well understood either.

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![Diagram of the synthesis of P antigen](image)

**Fig. 2** Schematic summary of the synthesis of P antigen. The α- or β-linkages are shown along with the informative numbers in the 1,3 or 1,4 glycosidic bonds.

- Glucose (Glc)
- Galactose (Gal)
- N-acetylgalactosamine (GalNAc)

---

**Tissue Distribution**

Expression of the P antigen has been studied in several species.\textsuperscript{20–22} Studies of mouse tissues show expression patterns similar to those of humans, although there are some differences.\textsuperscript{21}

The P antigen is expressed on a number of other cells in addition to RBCs, but various studies using different antibodies or methods have come to different conclusions about where it is expressed. Soluble P substance has also been detected in plasma.\textsuperscript{23,24} Small amounts of P\textsuperscript{k}, together with higher amounts of P, are present on intestinal epithelial cells.\textsuperscript{25} P antigen expression is also seen on megakaryocytes and fibroblasts but not on lymphocytes and granulocytes according to von dem Borne et al.,\textsuperscript{26} whereas Shevinsky et al.\textsuperscript{27} could not detect P on fibroblasts. In another study, P was detected in 11 of 16 investigated tissues, especially those of mesodermal origin.\textsuperscript{28} Placenta and fetal heart and kidney are other tissues where P is present.\textsuperscript{17} The P antigen is expressed on embryonal carcinoma cells and, according to Song et al.,\textsuperscript{29} is a possible initiator of signal transduction through AP-1 and CREB, associated with cell adhesion. High expression of the $B3GALNT1$ gene has been demonstrated in brain and heart, moderate expression in lung, placenta, and testis, and low expression in kidney, liver, spleen, and stomach.\textsuperscript{7}

**Disease Associations**

Parvovirus B19, which causes the so-called fifth disease, uses erythroid precursor cells expressing high levels of P antigen for its replication.\textsuperscript{30,31} Infection during pregnancy with B19 can give rise to fetal anemia and, in some cases, fetal loss as a result of inhibition of hematopoiesis.\textsuperscript{32} Paroxysmal cold hemoglobinuria, which can be seen in children after a viral infection, can be caused by an autoanti-P. This complement-fixing and cold-reactive antibody, also called Donath-Landsteiner antibody, lyses autologous P-positive erythrocytes.\textsuperscript{33} P-fimbriated uropathogenic *Escherichia coli* that express Pap-encoded adhesins bind to glycolipid structures containing the Gal4Gal motif and can cause urinary tract infections such as cystitis and pyelonephritis. This includes the P antigen and other globoseries antigens like P\textsuperscript{k}, as well as the neolactoseries P1 antigen.\textsuperscript{34,35}

**Related Antigens in the GLOB Collection**

**The LKE (Luke) Antigen**

The Luke (LKE, also known as SSEA-4 or monosialogalactosyldigloboside, MSGG) antigen was found in 1965\textsuperscript{36} and named LKE in 1986 after the first patient with anti-LKE. Since 1990, LKE belongs to the GLOB collection (ISBT no. 209). The antigen, which was given number 209003, is formed by sequential addition of a galactose (Gal) moiety and thereafter sialic acid (NeuAc) to the P antigen to form
The PX2 Antigen

The PX2 antigen, a high-prevalence antigen originally designated as the x₂ glycolipid, was first mentioned by Kannagi et al., and the structure was later characterized further by Thorn et al., who also found it elevated on RBCs with the p phenotype. It is formed by addition of a β3GalNAc to paragloboside, the same precursor used by the FUT1 enzyme to make H antigen or P₁ synthase to make P₁. There are also sialylated variants of x₂ described.

In individuals with the P₁,ₖ phenotype only anti-P is expected in plasma according to textbooks, but it was recently reported that another naturally occurring antibody specificity could give rise to a weak or variable crossmatch reactivity with p phenotype RBCs owing to their elevated amounts of x₂ glycolipid. At the same time, all P₁,ₖ and P₂,ₖ RBCs were compatible. In the absence of a functional β3GalNAc transferase because of B3GALNT1 mutations in P₁,ₖ and P₂,ₖ phenotype individuals, it is hypothesized that paragloboside can no longer be extended to form PX2 but this has yet to be proved. In the meantime, the PX2 antigen has been assigned to the GLOB collection (ISBT no. 209).

The biochemical and genetic relationship between the GLOB system and collection antigens discussed in this review is summarized in Figure 3.

Summary

The GLOB blood group system currently consists of only one antigen, P, or globoside, but is closely associated with the antigens in the P₁P₁K blood group system as well as the GLOB collection antigens. The P antigen can act as a receptor for various pathogens and anti-P can cause hemolytic transfusion reactions, spontaneous recurrent abortions, and autoimmune anemia as a result of lysis of erythroid progenitors.

References


43. Storry JR, Castillo L, Daniels G, et al. International Society of Blood Transfusion Working Party on red cell immunogenetics...

Åsa Hellberg, PhD (corresponding author), Coordinator at the Nordic Reference Laboratory for Genomic Blood Group Typing, Department of Clinical Immunology and Transfusion Medicine, University and Regional Laboratories, SE-221 85 Lund, Sweden, and Division of Hematology and Transfusion Medicine, Department of Laboratory Medicine, Lund University, SE-221 00 Lund, Sweden, Julia S. Westman, MSc, PhD student, Division of Hematology and Transfusion Medicine, Department of Laboratory Medicine, Lund University, Lund, Sweden, and Martin L. Olsson, MD, PhD, Medical Director of the Nordic Reference Laboratory for Genomic Blood Group Typing, Professor and Senior Consultant at the Department of Clinical Immunology and Transfusion Medicine, University and Regional Laboratories, and Division of Hematology and Transfusion Medicine, Department of Laboratory Medicine, Lund University, Lund, Sweden.
The antigens in the P1PK blood group system are carried on glycosphingolipids. The system currently includes three different antigens, P1, Pk, and NOR. The P1 antigen was discovered in 1927 by Landsteiner and Levine, and Pk and NOR were described in 1951 and 1982, respectively. As in the ABO system, naturally occurring antibodies of the immunoglobulin (Ig) M or IgG class, against the missing carbohydrate structures, can be present in the sera of people lacking the corresponding antigen. Anti-P1 is generally a weak and cold-reactive antibody not implicated in hemolytic transfusion reaction (HTR) or hemolytic disease of the fetus and newborn while Pk antibodies can cause HTR, and anti-NOR is regarded as a polyagglutinin. A higher frequency of miscarriage is seen in women with the rare phenotypes p, P1, and P2. Furthermore, the Pk and P1 antigens have wide tissue distributions and can act as host receptors for various pathogens and toxins. Why p individuals lack not only Pk and P expression but also P1 has been a longstanding enigma. Recently, it was shown that the same A4GALT-encoded galactosyltransferase synthesizes both the P1 and Pk antigens and that a polymorphism in a new exon in this gene predicts the P1 and P2 phenotypes. Immunohematology 2013;29:25–33.

Historical Aspects

In 1927, Landsteiner and Levine found antibodies to an antigen they called P (now known as the P1 antigen). In 1951, the Jay blood group “system” was discovered, containing one antigen, Tja, and its corresponding antibody, anti-Tja. Four years later, Sanger found a relationship between the P (P1) and the Jay systems. The P (P1) antigen was absent from all red blood cells (RBCs) with the rare Tj(a−) phenotype. The antigen P was then renamed to P1 and the Tj(a−) phenotype became the p (or PP1Pk null) phenotype. Anti-P was originally recognized in 1955 as a component of anti-Tja (now designated anti-PP1Pk), the mix of naturally occurring antibodies in sera of people with the p phenotype. The first individual lacking the P antigen was described in 1959 by Matson et al., and in the same paper the Pk antigen and anti-Pk were first mentioned. The authors also noted the association with the P blood group system.

Nevertheless, the relationship between the different antigens was not fully understood at that time. In the fifth edition of Blood Groups in Man, the authors declare “we began to feel lost in amazement at the complexity of the P system.” Indeed, this feeling has lingered (and still does) among many of us who work with these questions. However, the first paper explaining the relationship between the P1, Pk, and P antigens, as well as determining the biochemical structure of these glycolipids, was published in 1974 by Naiki et al. See Figure 1 for a schematic representation of the antigens discussed in this review.

Nomenclature

The history of nomenclature for the P1 and Pk antigens is complicated and sometimes confusing. The P1 antigen, originally called P, used to belong to the P blood group system and the Pk antigen to the GLOB collection (ISBT no. 209). However, the P antigen (globoside, Gb4) did not belong to the original P system, but has now been made the only antigen in the GLOB blood group system (ISBT no. 028). Now that it is clear that the A4GALT gene is responsible for both the P1 and Pk antigens (see later section), the Pk antigen has joined the P1 antigen and the system name has been changed to the P1PK blood group system (ISBT no. 003) to reflect the two
major antigens in it (Table 1). The NOR antigen was assigned to the P1PK system by the ISBT Working Party on Red Cell Immunogenetics and Blood Group Terminology at the 2012 annual meeting in Cancun (Table 1). The related antigens LKE and PX2, as well as P, are discussed in the GLOB blood group system review also published in this issue.

Table 1. The P1PK blood group system

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Blood group system</th>
<th>ISBT number</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1</td>
<td>P1PK</td>
<td>003001</td>
</tr>
<tr>
<td>P</td>
<td>P1PK</td>
<td>003003</td>
</tr>
<tr>
<td>NOR</td>
<td>P1PK</td>
<td>003004</td>
</tr>
</tbody>
</table>

003002 is obsolete.

The Pk antigen is also known as the Burkitt lymphoma antigen and has been classified as CD77. Yet another name, Gb3, is often used when the Pk structure is expressed on cells other than RBCs. This is in analogy with Gb4 being an alternative and more biochemically useful name for P.

Biochemistry

The P1PK and GLOB antigens are related and are structurally of a glycan nature. Depending on which carbohydrate residues are added to lactosylceramide (LacCer), different series of glycosphingolipids are formed. Glycosphingolipids were first described by Thudichum in 1884, and he named them after the inscrutable Egyptian Sphinx, as both the structure and the function were unknown at the time. These molecules consist of a sugar moiety with a lipid ceramide tail; they make up the outer leaflet of cell membranes together with phospholipids, cholesterol, and glycerolipids. Lipids are organised in microdomains such as glycosynapses and lipid rafts.

The P1PK antigens, as well as the GLOB system and collection antigens, are formed on the same precursor, LacCer, which is the most common precursor for glycosphingolipids in mammals and birds. Pk (other names include globotriaosylceramide, Gb3, ceramide trihexoside, and CTH) belongs to the globo-series, and P1 (also known as nLc5) to the neolacto/paraglobo-series.

The biochemistry of the P1 blood group antigen was partially elucidated by Morgan and Watkins in the 1960s by a series of experiments on hydatid cyst fluid from sheep infected by the tapeworm *Echinococcus granulosus*. They purified P1-specific components and showed that a glycoprotein containing the Galα1-4Galβ1-4GlcNAc trisaccharide reacted as a P1 determinant. Some years later, Marcus managed to extract P1 glycolipids from RBCs. In 1974, the Pk structure was identified as CTH by Naiki and Marcus.

The 4-α-galactosyltransferase (α4Gal-T/P1P_k synthase) catalyzes the transfer of galactose to the galactose residue on LacCer, producing the Pk antigen. In another pathway, the P1 antigen is formed by three sequential glycosylation reactions, the last one performed by the P1P_k synthase. Furthermore, other glycosyltransferases form additional blood group antigens associated with the P1PK/GLOB systems and collection, such as Forssman (FORS1), globo-H, and globo-A (Fig. 2).

It has been debated whether the Pk and P1 antigens exist on glycoproteins in the human RBC membrane, but according to Yang et al. glycolipids are the sole carriers of these antigens on RBCs.

Antigens and Antibodies in the System

Different combinations of the P1, P, and Pk antigens give rise to the following phenotypes: P1, P2, P1k, P2k, and p (Table 2). The prevalence of the P1 phenotype varies among different ethnic groups, ranging from 90 percent among Africans to 80 percent in whites down to 20 percent in Asians. On RBCs, P1 expression changes during fetal development. The antigen is found as early as week 12 but weakens during gestation. At birth the expression is low, and it takes up to 7 years before full expression is reached. The strength of the antigen expression can differ from one person to another, and it was proposed...
as early as 1953 to be dependent on dosage. The discovery of a $P^1/P^2$-predictive single-nucleotide polymorphism (SNP) did indeed prove this proposal to be correct, and it could be confirmed with traditional serology, antigen site density measurement by flow cytometry, and quantification of transcription levels by real-time polymerase chain reaction.

### Table 2. A summary of phenotypes and possible antibodies for the P1PK/GLOB blood groups

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>Prevalence</th>
<th>Antigens present on RBC</th>
<th>Antibodies in serum</th>
</tr>
</thead>
<tbody>
<tr>
<td>$P_1$</td>
<td>20–90%</td>
<td>$P_1, P_2, P$</td>
<td>Anti-$P_1^*$</td>
</tr>
<tr>
<td>$P_2$</td>
<td>10–80%</td>
<td>$P_2, P$</td>
<td>Anti-$P_2$</td>
</tr>
<tr>
<td>$p$</td>
<td>rare</td>
<td>$P_2, P$</td>
<td>Anti-$PP1P_2^*$</td>
</tr>
<tr>
<td>$P_1^*$</td>
<td>rare</td>
<td>$P_1, P_2^*$</td>
<td>Anti-$P_1^*$</td>
</tr>
<tr>
<td>$P_2^*$</td>
<td>rare</td>
<td>$P_2, P$</td>
<td>Anti-$P_1^*$</td>
</tr>
</tbody>
</table>

*Not always present, detectable, or both.

**Anti-PX2 can be present.

Individuals with the In(Lu) phenotype express lower amounts of $P_1$ antigen. In 2008 Singleton et al. found that the majority (21 of 24) of such individuals are heterozygous for mutations in their $EKL$ gene. This suggests that expression of $P^k$ and $P_1$ on RBCs may depend on binding of the erythroid transcription factor $EKL$ to the $A4GALT$ promoter.

The $P^k$ antigen was first thought to be a low-prevalence antigen, but later it was understood that nearly all the antigens are masked by addition of β3GalNAc to form globoside, the $P$ antigen, but later it was understood that nearly all the antigens are masked by addition of β3GalNAc to form globoside, the $P$ antigen, but later it was understood that nearly all the antigens are masked by addition of β3GalNAc to form globoside, but later it was understood that nearly all the antigens are masked by addition of β3GalNAc to form globoside, but later it was understood that nearly all the antigens are masked by addition of β3GalNAc to form globoside, or by the erythroid transcription factor $EKL$ to the $A4GALT$ promoter.

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The discovery of a $P^1/P^2$-predictive single-nucleotide polymorphism (SNP) did indeed prove this proposal to be correct, and it could be confirmed with traditional serology, antigen site density measurement by flow cytometry, and quantification of transcription levels by real-time polymerase chain reaction.

However, some antibodies against $P_1$ have been reported to react at 37°C, bind complement, and cause both immediate and delayed HTRs. Anti-$PP1P$ can cause HTR, but HDFN has not been reported. Instead, some of the women with anti-$PP1P$, or with anti-$P$ in the globoside-deficient null phenotypes of the GLOB blood group system, suffer from recurrent spontaneous abortions. The fetus as well as the newborn express low amounts of the $P_1, P$, and $P^k$ antigens, but the placenta shows high expression and is therefore a possible target of the antibodies that may be the cause of the miscarriages. It has been suggested that it is mainly the IgG anti-$P$ component in women with the $p$ phenotype that attacks the fetally derived cells in the placenta. In a group of 17 female Swedish $p$ individuals, 11 had experienced at least one spontaneous abortion.

The anti-$PP1P$ found in individuals with the $p$ phenotype was previously called anti-Tj*, named after Mrs. Tj in whose serum this antibody specificity was first found in association with a tumor.

NOR antigen expression, a low-prevalence polyagglutinable state, was first described in 1982 in a patient living in Norton, Virginia, hence the name. So far, it has only been found in two families, one in the United States and one in Poland. The RBCs of the initial patient were agglutinated by 75 percent of tested ABO-compatible sera but were not agglutinated by any lectin known to react with other types of polyagglutinable RBCs. The polyagglutination was inhibited by avian $P_1$ substance, and a possible relation to the P1PK blood group system was suggested. Family studies showed that the antigen was inherited and that anti-NOR is present in most adult human sera; it has therefore been called a polyagglutinin. It is unknown whether this kind of antibody is clinically significant, because transfusion of NOR+ blood is such a rare event.

### Genetics and Molecular Basis

#### The P1PK (A4GALT) Gene

The gene encoding the implicated 4-α-galactosyltransferase (a4Gal-T, EC 2.4.1.228) was cloned in 2000 by three independent research groups and was originally only found to give rise to the $P^k$ antigen. The gene is located on the long arm of chromosome 22 and consists of four exons with the whole coding region in the last exon (Fig. 3). This gene encodes a type II transmembrane glycoprotein with 353 amino acids and is highly conserved among different species. The protein sequence contains a characteristic DXD motif (amino acids 192–194), which is a conserved motif existing in nearly all glycosyltransferases.
proposed that this motif participates in the coordination of the metal ion and therefore also in the binding of the nucleotide part of the uridine diphosphate donor sugar. The crystal x-ray structure of the enzyme has not been solved yet, and the type of cation A4GALT required for its function has not been clarified. The promoter region of the gene has binding sequences for the transcription factor AP-1 (TGAGTCA), 160 bp upstream of the transcription start according to a computer search done by Hughes et al. In another study, it was shown that the promoter contains three binding sites for Sp1, four GC boxes, but no TATA or CCAAT boxes. However, a specific reason why this gene is expressed in erythroid tissues has not been reported.

A major enigma has been why the P1 antigen is always absent in the p phenotype. Different theories have been proposed. One model suggested that the same enzyme, α4Gal-T, is able to transfer galactosyl residues to both LacCer and paragloboside, but to use the latter as the acceptor, a regulatory protein is required. Another hypothesis suggested that two different enzymes exist, both of which must be inactivated to cause the p phenotype. This model was supposedly supported by a study showing that micromolar concentrations of P1, kidneys could synthesize both P1 and P2, whereas enzymes from P2 kidneys could only produce P2. A third model proposed a single gene with three alleles, one allele coding for a α4Gal-T using LacCer and paragloboside as the possible acceptors, one allele using LacCer only, and the third allele coding for an inactive form of the transferase.

No polymorphisms in the coding region of the A4GALT gene appeared to explain the P1/P2 phenotypes. Hence the theory with one gene encoding for both P1 and P2 was temporarily abandoned. Iwamura et al. proposed that transcriptional regulation, caused by two different polymorphisms in the 5′-regulatory region of A4GALT, might instead be the underlying reason for the P1/P2 phenotypes. However, these findings could not be verified in their own transcription assay or in two other independent studies.

In 2010, it was demonstrated that the A4GALT product could also synthesize P1 antigen. A genetic marker with which prediction of P1 versus P2 phenotype could be achieved was reported when a novel A4GALT transcript, containing exon 1 and a new exon, designated exon 2a, was discovered. This exon contains a P1- versus P2-associated polymorphism (42C/T), which also opens a short potential reading frame in P2 alleles. However, the mechanism by which this SNP operates is still unknown, even if 42T was shown to be correlated to lower A4GALT-mRNA levels. The authors hypothesize that either the new transcript, the hypothetical P2-related peptide, or the SNP in the genomic sequence itself may downregulate the transcription of A4GALT in P2 individuals. Alternatively, yet other polymorphisms closely linked in cis with this SNP could be involved. Several candidate SNPs occur in close proximity to exon 2a and 42C/T, as do potential binding sites for erythroid transcription factors. No matter which mechanism is active, typing for the 42C/T polymorphism correctly predicted the P1/P2 phenotype in 207 of 208 common Swedish blood donors, and full concordance was recently obtained also in 200 Asian donors (100 P1 and 100 P2).

To date, 29 mutations in 33 alleles of the A4GALT gene have been found to cause the p phenotype. For a complete list of these mutations and alleles, see the homepage of the International Society of Blood Transfusion (ISBT; www.isbtweb.org). Two silent polymorphisms, 903G>C (P301P) and 987G>A (T329T), as well as one missense mutation, 109A>G (M37V), with no apparent effect on the α4Gal-T, have also been documented. These polymorphisms were found to be organized into different haplotypes and linked to upstream and downstream SNPs. In addition, a few other noncritical polymorphisms have been found in combination with enzyme-crippling, p-associated mutations.

A missense mutation in the A4GALT gene causing an amino acid change, Gln211Glu, was found in individuals positive for the rare NOR antigen. This is believed to make the A4GALT-encoded enzyme add a galactose to the P antigen, thereby forming NOR. Thus, in addition to making P1 and P2 antigen, the Gln211Glu form of α4Gal-T also makes NOR. We sequenced the A4GALT genes of NOR+ family members and found that the NOR-specific SNP (631C>G) was linked to the P1-associated exon 2a SNP 42C. Further elongation of the NOR antigen (also called NOR1) can give rise to NORint and NOR2, two glycolipid structures not yet given blood group status by the ISBT.
**Tissue Distribution**

The expression of glycosphingolipids often shows a wide histological distribution but varies among tissues and species. Expression of the A4GALT-derived Pk and P1 antigens has been studied in several species. Studies on mouse tissues show expression patterns similar to those found in humans, although some differences have been noted.

The P1 structure is found as glycolipids, glycoproteins, or both in many organisms such as the nematode (Ascaris suum), tapeworm (Echinococcus granulosus), earthworm (Lumbricus terrestris), liver fluke (Fasciola hepatica), bacterium (Neisseira gonorrhoeae), and pigeon. The Pk antigen is also expressed in several strains of bacteria.

In humans, glycosphingolipids can be useful as surface markers of normal erythrocyte differentiation and of erythroleukemias. The Pk and P1 antigens are expressed on a number of other cells in addition to RBCs, but various studies using different antibodies or methods have come to different conclusions about where they are expressed. Pk has been detected in plasma, but no such reports about P1 in plasma or about Pk and P1 in secretions are available.

The P1 antigen is expressed on B lymphocytes, granulocytes, and monocytes. The Pk antigen has been found on all leucocytes (except NK cells), fibroblasts, platelets, and smooth muscle cells of the digestive tract and urogenital system, and is a differentiation antigen expressed on a subset of tonsillar B cells in the germinal center. High expression of Pk in the kidney has been implicated in susceptibility to hemolytic uremic syndrome (HUS), further discussed in the section on disease associations. The mechanism behind the high renal expression might be related to enhanced A4GALT gene transcription and reduced α-galactosidase gene activity. Recently, it has also been shown that small amounts of Pk together with higher amounts of P are present on intestinal epithelial cells.

Northern blot studies of human organs showed high expression of the A4GALT gene in kidney and heart in one study, while another report described high expression in spleen, liver, testis, and placenta, in addition to kidney and heart.

**Disease Associations**

The Pk and P1 antigens can act as membrane receptors for several pathogens and toxins, summarized in Table 3.

<table>
<thead>
<tr>
<th>Pathogen/toxin</th>
<th>Disease</th>
<th>Antigen involved</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>HIV</td>
<td>AIDS</td>
<td>Pk</td>
<td>76,77</td>
</tr>
<tr>
<td>Uropathogenic <em>Escherichia coli</em></td>
<td>UTI</td>
<td>Pk, P1</td>
<td>78,79</td>
</tr>
<tr>
<td><em>Streptococcus suis</em></td>
<td>Meningitis</td>
<td>Pk, P1</td>
<td>80</td>
</tr>
<tr>
<td><em>Shigella dysenteriae</em> (Shiga toxin)</td>
<td>Dysentery</td>
<td>Pk</td>
<td>81</td>
</tr>
<tr>
<td><em>Escherichia coli</em> O157 (Stx 1/2)</td>
<td>HUS, hemorrhagic colitis</td>
<td>Pk</td>
<td>81,82</td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em> (PA-IL lectin)</td>
<td>Opportunistic human pathogen</td>
<td>Pk, P1</td>
<td>83</td>
</tr>
</tbody>
</table>

HIV = human immunodeficiency virus; AIDS = acquired immune deficiency syndrome; UTI = urinary tract infection; HUS = hemolytic uremic syndrome.

**Viruses**

Although initial studies implied a facilitating role for Pk in human immunodeficiency virus (HIV) infection, recent work has suggested that Pk is protective when accumulated owing to reduced activity of α-galactosidase A in Fabry disease, an X-linked lysosomal storage disorder. In addition, a soluble analogue of Pk prevents HIV infection in vitro. Another study showed that peripheral blood mononuclear cells (PBMCs) with Pk phenotype were highly resistant to infection, whereas PBMCs with the p phenotype showed increased susceptibility to infection. Furthermore, Pk-liposome fusion into the Pk-deficient Jurkat T-cell line reduced productive X4 HIV-1 infection, as did overexpression of Pk synthase. Accordingly, siRNA silencing of the Pk synthase gene increased the cells’ HIV susceptibility.

**Bacteria**

Uropathogenic *Escherichia coli* expressing pap-encoded PapG adhesins bind to Pk and P1, and both the *Streptococcus suis* adhesin and the PA-IL lectin from *Pseudomonas aeruginosa* use Pk (Gb3) and P1 as receptors. Furthermore, Pk is the receptor for Shiga toxin from *Shigella dysenteriae* (Stx) or certain *E. coli* strains (Stx1 and Stx2) on renal epithelium, platelets, and endothelium.

A disease connected to the Pk antigen is Fabry disease, in which deficiency of the lysosomal enzyme α-galactosidase A causes accumulation of sphingolipids, mainly Pk, in some cell types and body fluids. It has been shown that mice with Fabry disease are protected against Stx from enterohemorrhagic *E. coli* (EHEC). These data are surprising because Pk is the cellular receptor for Stx and therefore a higher sensitivity would be expected. The authors hypothesize that the excess Pk can work as a toxin sink, which allows the toxin to bind to Pk in tissues that normally do not have high expression.
and cannot be affected by the toxin. EHEC infection can induce HUS, which leads to hemolytic anemia, renal failure, and thrombocytopenia. According to Furukawa et al., the mechanism behind the thrombocytopenia might be that Stx binds to P in immature megakaryoblasts and induces their apoptosis, leading to the restraint of platelet production in the bone marrow. The P antigen has been shown to mediate apoptotic signals after the binding of both Stx and anti-P (CD77 monoclonal antibody). These ligands trigger two completely different apoptotic pathways, one caspase- and mitochondria-dependent and one reactive oxygen species–dependent. It has also been shown that patients with HUS have lower levels of P glycolipid in their sera compared with a healthy control group. These authors propose that circulating Stx could bind to P glycolipids in sera during infection, which may reduce the amount of Stx binding to the target cells. Consequently, patients with low serum levels of P would have a higher susceptibility to EHEC infections. Another study states that only P and not P1, as earlier believed, is the receptor for Stx, and mice without P lose sensitivity to Stx.

**Cancer**

Altered glycosylation patterns of glycosphingolipids such as neoexpression, underexpression, or overexpression are characteristic of cancer cells. One example is the first described p individual (lacking P, P, and P1 antigens), who had a gastric tumor that expressed P1 antigen. Levine proposed that the antibodies made against the P, P, and P1 antigens prevented further growth of the tumor. Altered expression of P antigen has also been described in ovarian carcinomas, colon cancer, breast cancer, and B-cell lymphomas. It has even been suggested that Stx, which specifically binds to P, could be used as a targeted cancer therapy.

**Summary**

The history of the P1PK blood group system is complex and not easy to grasp because of several changes of nomenclature. In addition, the biochemical background and genetic basis have caused long debates, and even today many basic questions remain to be solved. However, step by step the biochemical and genetic basis underlying the antigens expressed in this system has been revealed. The most recent but certainly not the final step came when it was clarified that the A4GALT gene is responsible not only for P expression but also for P1 and even NOR expression. As a result, the P and later the NOR antigen joined the P1 antigen, and the system name was changed to P1PK.

**References**


58. Hellberg Å, Chester MA, Olsson ML. Two previously proposed P1/P2-differentiating and nine novel polymorphisms at the A4GALT (Pk) locus do not correlate with the presence of the P1 blood group antigen. BMC Genet 2005;6:49.

59. Tilley L, Green C, Danies G. Sequence variation in the 5’ untranslated region of the human A4GALT gene is associated with, but does not define, the P1 blood-group polymorphism. Vox Sang 2006;90:198–203.


In this issue of *Immunohematology*, we introduce the New Blood Group Allele Report. This is a mechanism for describing a blood group allele that has not been described in a peer-reviewed publication. Genetic variation in blood groups is extensive, and although the Human Genome Project and HapMap Projects have been completed, there continue to be new discoveries of blood group alleles associated with a serologic phenotype and, in some cases, found in individuals with an antibody to the associated antigen. Oftentimes, these alleles are shared with the blood banking and molecular immunohematology community through abstracts at scientific meetings. However, in many cases the discovery of these alleles is not subjected to peer review nor published in a journal, where it can be found via a literature search (PubMed). Another avenue to communicate the new finding is through the International Society of Blood Transfusion (ISBT) Working Party on Blood Group Allele Terminology (http://www.isbtweb.org/working-parties/red-cell-immunogenetics-and-blood-group-terminology/blood-group-terminology/blood-group-allele-terminology/), where new alleles can be assigned an allele number, using a standard nomenclature format, and posted on a Web site for easy reference. However, this venue does not capture important supporting information about the allele, such as in what ethnic group it was discovered, what methods were used to identify it, and any references that include more detail.

With the New Blood Group Allele Report, *Immunohematology* is filling a need to communicate these new alleles to the community. The format for these submissions is designed to be straightforward, capturing parameters about the new allele that are pertinent, and to allow the information to be shared efficiently. Refer to the Instructions for Authors page. We hope our readers will use this forum to share their discoveries.
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Manuscripts

The editorial staff of *Immunohematology* welcomes manuscripts pertaining to blood group serology and education for consideration for publication. We are especially interested in case reports, papers on platelet and white cell serology, scientific articles covering original investigations, and papers on new methods for use in the blood bank. For instructions for scientific articles, case reports, and review articles, see Instructions for Authors in every issue of *Immunohematology* or e-mail a request to immuno@redcross.org. Include fax and phone numbers and e-mail address with all manuscripts and correspondence. E-mail all manuscripts to immuno@redcross.org

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*Immunohematology* will publish classified ads and announcements (SBB schools, meetings, symposia, etc.) without charge.

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Rush University is fully accredited by the Higher Learning Commission [HLC] of the North Central Association of Colleges and Schools and the SBB Certificate Program is accredited by the Commission on Accreditation of Allied Health Education Programs (CAAHEP).

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- Provide technical and scientific training in transfusion medicine
- Conduct research in transfusion medicine

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Revised May 2012
I. GENERAL INSTRUCTIONS

Before submitting a manuscript, consult current issues of Immunohematology for style. Number the pages consecutively, beginning with the title page.

II. SCIENTIFIC ARTICLE, REVIEW, OR CASE REPORT WITH LITERATURE REVIEW

A. Each component of the manuscript must start on a new page in the following order:
1. Title page
2. Abstract
3. Text
4. Acknowledgments
5. References
6. Author information
7. Tables
8. Figures

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1. Title page
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   a. One paragraph, no longer than 300 words
   b. Purpose, methods, findings, and conclusion of study

3. Key words
   a. List under abstract

4. Text (serial pages): Most manuscripts can usually, but not necessarily, be divided into sections (as described below). Survey results and review papers may need individualized sections
   a. Introduction — Purpose and rationale for study, including pertinent background references
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2. Annotated conference proceedings

B. Preparation of manuscript

1. Title page
   a. Capitalize first word of title.
   b. Initials and last name of each author (no degrees; all CAPs)

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   a. Case should be written as progressive disclosure and may include the following headings, as appropriate
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      ii. Immunohematologic Evaluation and Results: Serology and molecular testing
      iii. Interpretation: Include interpretation of laboratory results, correlating with clinical findings
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IV. LETTER TO THE EDITOR

A. Preparation

1. Heading (To the Editor)
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4. Author(s) (type flush right; for first author: name, degree, institution, address [including city, state, Zip code and country]; for other authors: name, degree, institution, city and state)
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Send all manuscripts by e-mail to immuno@redcross.org
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C. Text
   1. Case Report
      i. Clinical and immunohematologic data
      ii. Race/ethnicity and country of origin of proband, if known
   2. Materials and Methods
      Description of appropriate controls, procedures, methods, equipment, reagents, etc. Equipment and reagents should be identified in parentheses by model or lot and manufacturer’s name, city, and state. Do not use patient names or hospital numbers.
   3. Results
      Complete the Table Below:

      | Phenotype | Allele Name | Nucleotide(s) | Exon(s) | Amino Acid(s) | Allele Detail | References |
      |-----------|-------------|---------------|---------|---------------|---------------|------------|
      | e weak    | RHCE*01.01  | 48G>C         | 1       | Trp16Cys      | RHCE*ce48C    | 1          |

Column 1: Describe the immunohematologic phenotype (ex. weak or negative for an antigen).
Column 2: List the allele name or provisional allele name.
Column 3: List the nucleotide number and the change, using the reference sequence (see ISBT Blood Group Allele Terminology Pages for reference sequence ID).
Column 4: List the exons where changes in nucleotide sequence were detected.
Column 5: List the amino acids that are predicted to be changed, using the three-letter amino acid code.
Column 6: List the non-consensus nucleotides after the gene name and asterisk.
Column 7: If this allele was described in a meeting abstract, please assign a reference number and list in the Reference section.

4. Additional Information
   i. Indicate whether the variant is listed in the dbSNP database (http://www.ncbi.nlm.nih.gov/snp/); if so, provide rs number and any population frequency information, if available.
   ii. Indicate whether the authors performed any population screening and if so, what the allele and genotype frequencies were.
   iii. Indicate whether the authors developed a genotyping assay to screen for this variant and if so, describe in detail here.
   iv. Indicate whether this variant was found associated with other variants already reported (ex. RHCE*ce48C,1025T is often linked to RHD*DVa-2)

D. Acknowledgments

E. References

F. Author Information
   List first name, middle initial, last name, highest degree, position held, institution and department, and complete address (including ZIP code) for all authors. List country when applicable.
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